論 文 の 内 容 の 要 旨

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平成 23 年度博士課程進学
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Studies on the transcriptional regulatory mechanisms of myosin heavy chain genes expression during skeletal muscle development in fish

（魚類骨格筋形成におけるミオシン重鎖遺伝子の転写制御機構に関する研究）

Vertebrate skeletal muscle consists of heterogeneous tissues including various types of muscle fibers and the fiber-type specification is crucial for muscle development. Myosin is the major structural and functional muscle protein in the contractile apparatus. Terminal differentiation and specification of various types of muscle cells during development require specific expression of myosin heavy chain (MYH) isoforms; thereby MYH is thought to be a key molecule on the functional diversity of muscles. As in mammalian skeletal muscles, the members of the MYH genes (*MYH*s) of fish are expressed in a complex, sequential fashion during development and growth. Although a considerable progress has been made in elucidating the molecular genetics underlying the development-dependent, muscle-specific expression of *MYH*s in mammals, little is currently known regarding comparable issues in fish. In this study, the 5'-flanking sequences of development-dependent, fast- and slow-type *MYH*s of torafugu *Takifugu rubripes*, $MYH_{M743\text{-}2}$ and $MYH_{M86\text{-}2}$, respectively, were cloned and analyzed for their transcriptional regulation in zebrafish *Danio rerio* embryos using transient and transgenic approaches. The present study also focuses on the expressional regulation of *MYH*s, which are supposed to be associated with hyperplastic muscle growth in fish.

1. Transcriptional regulation of fast muscle-specific $MYH_{M743\text{-}2}$ expression

$MYH_{M743\text{-}2}$ is highly expressed in fast muscle fibers of torafugu embryos and larvae. When the 2075-bp 5'-flanking region of $MYH_{M743\text{-}2}$ was fused to the *EGFP* encoding enhanced green fluorescence protein in pT2AL200R1150G vector, the construct could induce muscle-specific expression of EGFP in zebrafish embryos. In most cases, the EGFP expression was detected at 1 day post fertilization (dpf) in the somite formation stage. After hatching at 2dpf, EGFP continued to be expressed in the whole myotome of larvae as well as in jaw, eye and pectoral fin muscles. The expression of the transgene in embryos and larvae from the stable transgenic line of

zebrafish was consistent with that of zebrafish embryos microinjected the 2075 bp construct. While the injected 2075 bp construct showed EGFP expression in both fast and slow muscle fibers of larvae as revealed by immunohistochemical analysis, embryos from the stable transgenic line expressed EGFP in fast muscle fibers only. These results clearly demonstrated that the 2075 bp 5'-flanking region of $MYH_{M743\text{-}2}$ contains essential *cis*-regulatory sequences for myogenesis that are conserved among torafugu and zebrafish.

Using the MatInspector and TFsearch programs, the 2075-bp 5'-flanking region from the start codon of $MYH_{M743\text{-}2}$ was found to contain putative transcription factor-binding sites for myocyte enhancing factor 2 (MEF2), myogenic determining factor (MyoD) and serum response factor (SRF), all of which have been implicated in the expressional regulation of muscle-specific genes during development. Embryos microinjected a modified 2075 bp construct, where the total three SRF binding sites were deleted, showed a significantly reduced EGFP expression. However, the deletion of all SRF binding sites failed to eliminate $MYH_{M743\text{-}2}$ expression completely. Moreover, the minimum 468-bp basal promoter region from the start codon containing the third SRF binding site from the 5'-end was found to direct EGFP expression in the myotomal compartment. Mutations of this SRF binding site in the 468-bp construct also reduced $MYH_{M743\text{-}2}$ expression in the myotomal compartments, suggesting that this site is necessary for mediating the basal expression of $MYH_{M743\text{-}2}$. In this regard, the expression of two zebrafish SRFs, *srf1* and *srf2*, was observed as early as at 2 hours post fertilization and continued to be expressed throughout the successive developmental stages. Such expression pattern of *srf*s was consistent with their potential roles in regulating $MYH_{M743\text{-}2}$ expression in zebrafish embryos.

There were three MyoD and eight MEF2 binding sites within the 2075-bp 5'-flanking region of $MYH_{M743\text{-}2}$. Functional analyses revealed that deletions of multiple sites of these factors significantly reduced the $MYH_{M743\text{-}2}$ promoter activity in skeletal muscles. Such reductions were also validated by real-time PCR analysis. Deletion mutation analyses also suggested that MyoD, but not MEF2, binding sites are crucial for $MYH_{M743\text{-}2}$ promoter activity in craniofacial and pectoral fin muscles. However, the deletion of all MyoD- or MEF2-binding sites failed to eliminate $MYH_{M743\text{-}2}$ expression completely. Taken together, it is indicated that multiple transcription factors including SRF, MyoD and MEF2 participate in the expression of $MYH_{M743\text{-}2}$.

Torafugu $MYH_{M743\text{-}2}$ showed homology over 55% on the rVISTA analysis in the 5' flanking region from −664 to −364 bp with the corresponding region of a green spotted pufferfish *Tetraodon nigroviridis* orthologous gene, *MYH13*. Embryos microinjected the 2075-bp construct excluding this conserved region completely abolished the EGFP expression, suggesting that the conserved regulatory *cis*-elements in this region regulate the transcriptional activity of $MYH_{M743\text{-}2}$. Further studies are required to precisely map responsible *cis*-elements in this region.

2. Transcriptional regulation of slow muscle-specific $MYH_{M86\text{-}2}$ expression

$MYH_{M86\text{-}2}$ transcripts are highly expressed in slow muscle fibers of torafugu embryos and larvae. To examine whether or not the 5'-flanking sequence of torafugu $MYH_{M86\text{-}2}$ would function similarly in zebrafish, we constructed a reporter vector containing the 2614 bp 5'-flanking region fused to *EGFP* in pT2AL200R1150G vector. The microinjection of the 2614 bp construct in zebrafish embryos exhibited EGFP expression in slow muscle fibers at 1dpf and continued to be expressed in the whole myotomal region in larvae at 2dpf. The transient superficial slow muscle-specific expressions of $MYH_{M86\text{-}2}$ were further confirmed by generating stable transgenic zebrafish lines. Apart from the slow muscle-specific expression, both the endogenous $MYH_{M86\text{-}2}$ and the reporter

gene were also found to be expressed in pectoral fin muscles. These data suggest that the 2614-bp 5'-flaking region contained the necessary regulatory elements for $MYH_{M86\text{-}2}$ expression in slow muscle fibers.

Hedgehog signaling is important for slow muscle development in vertebrates. To further characterize EGFP expression in slow muscle fibers, stable transgenic line zebrafish embryos, carrying the transgene $MYH_{M86\text{-}2}$: *EGFP*, were treated with cyclopamine (2.5, 5 and 10 μg/ml), a well-known hedgehog signaling inhibitor. Depending on the dose, cyclopamine-treated embryos showed reduction or complete elimination of EGFP expression in slow muscle fibers, suggesting that $MYH_{M86\text{-}2}$ promoter activity depends on the hedgehog signaling.

To identify the key regulatory region for the slow muscle-specific expression, a deletion analysis was performed within the 5.5-kb 5'-flanking region of $MYH_{M86\text{-}2}$. The deletion of the 5'-flanking region up to -2614 bp upstream of the $MYH_{M86\text{-}2}$ promoter had no or marginal effect on the activity and slow muscle-specificity of the promoter. The deletion of a distal 500 bp region (-2000/-1500) caused ectopic $MYH_{M86\text{-}2}$ expression in fast muscle fibers. *In silico* analysis revealed five putative Sox6 binding sites which spanned the regions at -1953/-1947 (Sox6_1), -1525/-1519 (Sox6_2), -808/-802 (Sox6_3), -792/-786 (Sox6_4) and -41/-35 (Sox6_5) in the 2,614 bp 5'-flanking region of $MYH_{M86\text{-}2}$, including the first two Sox6 binding sites (Sox6_1 and Sox6_2) in the 500 bp distal region. Notably, the deletion of these Sox6 binding sites (except Sox6_5) individually or in various combinations caused ectopic EGFP expression in the fast muscle fibers. In mice and zebrafish, Sox6 directly represses the transcription of slow muscle fiber-enriched genes by binding to conserved *cis*-regulatory elements. In consistent with such finding, the deletion of these Sox6 binding elements (except Sox6_5) significantly increased the reporter gene mRNA expression. Thus, in addition to fast muscle-specific repressive function, Sox6 elements may also function as a transcriptional suppressor of $MYH_{M86\text{-}2}$ promoter activity.

Promoter dissection study also showed that the deletion of 614 bp spanning the region from -2614 to -2001 significantly reduced the number of EGFP expressing muscle fibers. It suggests that 614 bp region may contain positive *cis*-elements required for slow muscle-specific expression of $MYH_{M86\text{-}2}$. Several studies on various model system have reported that calcineurin/nuclear factor of activated T cells (NFAT) pathways appear to be involved in the transcriptional regulation of slow muscle-specific gene expression including those of slow *MYH*s. *In silico* analysis identified four putative NFAT binding sites spanning the region at -2258/-2254 (NFAT_1), -1968/-1964 (NFAT_2), -1604/-1600 (NFAT_3) and -306/-302 (NFAT_4) bp in the 2,614 bp 5'-flanking region of $MYH_{M86\text{-}2}$, including the first one (NFAT_1) in the 614 bp distal region. Embryos microinjected these NFAT binding sites deletion constructs (except NFAT_2) showed significantly reduced EGFP expression in slow muscle fibers, and elimination of multiple and all the sites of NFAT elements showed more pronounced effects. However, the deletion of all NFAT binding sites could not completely abolish the EGFP expression, suggesting that *cis*-elements other than NFAT binding sites require for directing the $MYH_{M86\text{-}2}$ expression.

Members of myogenic regulatory factors (MyoD, Myogenin, myf5 and MRF4) and MEF2 have all been shown to regulate the transcription of numerous muscle-specific genes. Analysis of the 2614 bp 5'-flanking region of $MYH_{M86\text{-}2}$ by Genomatix MatInspector program identified two putative MyoD and three MEF2 binding sites. The deletion of both two MyoD binding sites did not significantly reduce EGFP expression percentages in slow muscle fibers. However, the deletion of multiple MEF2 binding sites significantly reduced EGFP expression in slow muscle fibers. These data suggest that MEF2, but not MyoD, participates in the transcriptional regulation of $MYH_{M86\text{-}2}$ expression.

3. *MYH*s expression associated with hyperplastic muscle growth in fish

The relationship of *MYH*s expression with muscle fiber formation has not been well understood in fish. In torafugu, the transcript of $MYH_{M2528\text{-}1}$ appeared in embryos at 6 dpf and continued to be expressed in later embryonic and larval development successively as well as in adult fast and slow skeletal muscles. The dorso-ventral extremes of larval myotome are considered as the main growth engines for recruitment of new fibers by stratified hyperplasia. Interestingly, the transcripts of $MYH_{M2528\text{-}1}$ were localized specifically in the dorsal and ventral extremes of larval myotome, suggesting its involvement in stratified hyperplasia. Apart from the involvement in stratified hyperplasia in torafugu larvae, *in situ* hybridization study showed that $MYH_{M2528\text{-}1}$ is also involved in mosaic hyperplasia in juvenile torafugu where its transcripts were expressed in fast fibers with small diameters as well as inner parts of superficial slow muscle fibers.

In zebrafish, recent studies showed that two *MYH*s, *myhz1* and *myhz2*, are strongly up-regulated by 8-19 folds at the stage of active myotube production, suggesting that they are involved in muscle hyperplasia. There were 3 paralogues of fast skeletal muscle-specific *myhz1*s such as *myhz1.1*, *myhz1.2* and *myhz1.3*. In torafugu, only $MYH_{M2528\text{-}1}$ is found to be involved in hyperplastic muscle growth. But in zebrafish, the functional significance of the combined involvement of *myhz1* and *myhz2* in hyperplasia are unknown. Then, we constructed reporter vectors containing the 4 kb and 3kb 5'-flanking region for *myhz1*s (*myhz1.2* and *myhz1.3*; amplification of *myhz1.1* was not successful) and *myhz2*, respectively. Interestingly, zebrafish embryos microinjected the 4 kb construct of *myhz1.2* and *myhz1.3* showed restricted EGFP expression in anterior somites and the 3 kb construct of *myhz2* did in the posterior tail somites of the skeletal muscle. Such posterior muscle-specific expression patterns of *myhz2* were further confirmed by generating stable transgenic line zebrafish, but the establishment of stable transgenic lines for the *myhz1* isoforms has not been successful. These results suggest that the expression patterns of *myhz1* and *myhz2* are complementary to each other. Previous study showed the association between *myhz2* expression and tail development by analyzing the spatial *myhz2* mRNA distribution pattern in *spadetail* (*spt*) and *no tail* (*ntl*) mutant zebrafish embryos. Therefore, we speculate that *myhz2* may be regulated by T-box factor (Tbx). Our study demonstrated that Tbx binding sites govern the restricted expression of *myhz2* in tail somites region of zebrafish. However, more conclusive evidence must be derived from analyses of the *myhz2* promoter gene in the future.

Conclusion

The present study provides a greater insight about the transcriptional regulatory mechanisms involved in *MYH*s expression during skeletal muscle development in fish. This study is the first report about the transcriptional regulatory mechanisms involved in the slow muscle-specific *MYH* expression in fish. A greater understanding of the factors responsible for the transcriptional regulation of *MYH*s expression in different muscle fibers-types would provides a greater insight into how these muscle fibers are established and maintained in fish. In this study, the *cis*-elements are identified by deletions and mutations analyses in zebrafish embryos. However, a putative consensus binding sequence may not necessarily bind the predicted transcription factors and deletions of putative binding sites may affect the binding of other transcription factors. Therefore, further studies are needed to investigate whether and how the predicted transcription factors bind to the promoters and whether the predicted transcription factors interact with other cofactors to activate *MYH*s expression in fish.